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Bacterial ghosts as novel advanced drug delivery systems: antiproliferative activity of loaded doxorubicin in human Caco-2 cells

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Abstract

Systemic application of anticancer drugs often causes severe toxic side effects. To reduce the undesired effects, advanced drug delivery systems are needed which are based on specific cell targeting vehicles. In this study, bacterial ghosts from *Mannheimia haemolytica* were used for site-specific delivery of doxorubicin (DOX) to human colorectal adenocarcinoma cells (Caco-2). Bacterial ghosts are non-denatured envelopes of Gram-negative bacteria with fully intact surface structures for specific attachment to mammalian cells. The in vitro release profile of DOX ghosts demonstrated that the loaded drug was non-covalently associated with the bacterial ghosts and that the drug delivery vehicles themselves represent a slow release system. Adherence studies showed that the *M. haemolytica* ghosts more efficiently than *E. coli* ghosts targeted the Caco-2 cells and released the loaded DOX within the cells. Cytotoxicity assays revealed that the DOX ghosts exhibited potent antiproliferative activities on Caco-2 cells as the DOX associated with ghosts was two magnitude of orders more cytotoxic than free DOX provided in the medium at the same concentrations. Notably, a significant reduction in the cell viability was measured with DOX ghosts at low DOX concentrations, which had no inhibitory effect when applied as free DOX after incubation for 16 h or when applied at higher concentrations for only 10 min to the cells. As the higher antiproliferative effects of DOX on Caco-2 cells were mediated by the specific drug targeting properties of the bacterial ghosts, the bacterial ghost system represents a novel platform for advanced drug delivery.

Keywords: Drug targeting; Doxorubicin; Bacterial ghosts; Colorectal adenocarcinoma; Caco-2; Platform technology

1. Introduction

Doxorubicin (DOX, adriamycin) is an antineoplastic agent that is active against a wide range of human and animal cancers. These include lymphoma, leukemia, mammary and thyroid carcinomas, lung and ovarian cancer, and sarcomas of bones and soft tissue [20]. This non-cell cycle-specific antitumor antibiotic intercalates with DNA and inhibits topoisomerase II. As a result, DOX is highly efficient against rapid replicating cancer cells [20]. However, severe toxic side effects have been reported for the use of this drug. The most prominent side effects include cardiotoxicity, irreversible dilated cardiomyopathy, cardiac

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Exhibit A

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Bacterial Ghost Technology for Pesticide Delivery

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Bacterial ghosts are nondenaturated empty cell envelopes of Gram-negative bacteria produced by E-mediated lysis. Such envelopes from the plant-adhering bacterium *Pectobacterium cypripedii* were tested for their ability to adhere to plant material and to be used as carriers for pesticide delivery. We show, using fluorescence-labeled *P. cypripedii* ghosts, that depending on the target plants 55 or 10% (rice or soya, respectively) of the applied bacterial ghosts was retained on the leaves after heavy simulated rain (84 mm). Furthermore, the bacterial ghosts could be loaded with the lipophilic triazole fungicide tebuconazole. In subsequent plant experiments in the glass house, the efficacy of the loaded bacterial ghost for resistance to rainfall and the protective and curative effects against the pathogens *Erysiphe graminis*, *Leptosphaeria nodorum*, and *Pyrenophora teres* on barley and wheat and against *Sphaerotheca fuliginea* on cucumber were tested. The bacterial ghosts were compared primarily with a commercial tebuconazole formulation, a wettable powder, as it has similar physical characteristics. The comparison revealed similar effects and showed consistently higher or comparable efficacy against the pathogens. The standard operational comparison with the most protective, cereal specific emulsion of oil in water displayed that the bacterial ghosts had equal to or lower efficacy than the emulsion. This study confirmed the potential of bacterial ghost platform technology as a new alternative carrier system for pesticides.

KEYWORDS: **Bacterial ghosts; carrier; pesticide; formulation; plant; adhesion; delivery system**

INTRODUCTION

Bacterial ghosts, which represent empty cell envelopes of Gram-negative bacteria, have been applied successfully as vaccine candidates (*1*) or as potential drug carriers (*2*, *3*). In this study, for the first time, bacterial ghosts were tested for their application as carrier and targeting vehicles for agricultural plants. Certain bacteria living on plant surfaces possess special adhesive capabilities. In this investigation, we used *Pectobacterium cypripedii*. This bacterium belongs to the previously described group of *Erwinia cypripedii* that were originally isolated from orchids (*Cypripedium* sp.) (*4*). They are ketogenic bacteria and members of the family *Enterobacteriaceae* of the γ-subgroup of *Proteobacteria*. The original phylogenic position of the genera has been amended to *Pectobacterium* (*5*). Bacterial ghosts are intact, nondenaturated, bacterial envelopes, which have been produced from various Gram-negative bacteria (*6*). The ghosts are produced by the controlled expression of the plasmid-encoded lysis gene *E* of bacteriophage φX174, which is under the control of either the temperature sensitive λ-promoted cI857 repressor system (*7*) or the chemically inducible promoter system (*8*). After gene *E* expression, the cytoplasmic content is expelled through an E specific transmembrane tunnel that penetrates the inner and outer membranes of Gram-negative bacteria, resulting in empty, nondenatured, bacterial cell envelopes (*9*). These envelopes can be used as carriers for specific drug delivery, and the aim of the present study was to investigate the feasibility of their exploitation in pesticide application.

Reduction in pesticide use is a major goal in agriculture as it addresses increasing environmental, health, and consumer concerns (*10*, *11*). Much research is therefore directed toward new types of pesticides and formulations with properties of greater efficiency that allow reductions in the amount and frequency of application (*13*). The fungicide tebuconazole [1-(4-chlorophenyl)-4,4-dimethyl-3-[1,2,4]triazole-1-ylmethyl-pentan-3-ol] is characterized by a high level of efficacy against a wide range of pathogens causing, e.g., powdery mildew, leaf blotch, and rust diseases. It has a systemic mode of action and interferes with the metabolism of the fungal pathogen by inhibiting sterol biosynthesis (*12*). Tebuconazole is registered for use on more

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Exhibit B

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GENE DELIVERY

Immobilization of plasmid DNA in bacterial ghosts

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Abstract

The development of novel delivery vehicles is crucial for the improvement of DNA vaccine efficiency. In this report, we describe a new platform technology, which is based on the immobilization of plasmid DNA in the cytoplasmic membrane of a bacterial carrier. This technology retains plasmid DNA (Self Immobilizing Plasmid, pSIP) in the host envelope complex due to a specific protein/DNA interaction during and after protein E-mediated lysis. The resulting bacterial ghosts (empty bacterial envelopes) loaded with pDNA were analyzed in detail by real time PCR assays. We could verify that pSIP plasmids were retained in the pellets of lysed *Escherichia coli* cultures indicating that they are efficiently anchored in the inner membrane of bacterial ghosts. In contrast, a high percentage of control plasmids that lack essential features of the self-immobilization system were expelled in the culture broth during the lysis process. We believe that the combination of this plasmid immobilization procedure and the protein E-mediated lysis technology represents an efficient in vivo technique for the production of non-living DNA carrier vehicles. In conclusion, we present a "self-loading", non-living bacterial DNA delivery vector for vaccination endowed with intrinsic adjuvant properties of the Gram-negative bacterial cell envelope.

Keywords: Bacterial ghosts; Self-immobilization; DNA carrier vehicle; DNA delivery system; Gene transfer

1. Introduction

The ability of DNA vaccination to induce immune response has been shown in a range of infectious disease models [1]. However, using naked DNA, it has become apparent that high doses and/or multiple immunizations are required to induce immune responses in larger animals and humans [2,3]. Therefore much effort is now focused on increasing the

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Exhibit C

The Journal of Immunology

Bacterial Ghosts Are an Efficient Delivery System for DNA Vaccines[1]

Thomas Ebensen,[2]* Susanne Paukner,[2†‡] Claudia Link,* Pavol Kudela,[¶§] Carola de Domenico,* Werner Lubitz,[†‡] and Carlos A. Guzmán[3]*

Mass implementation of DNA vaccines is hindered by the requirement of high plasmid dosages and poor immunogenicity. We evaluated the capacity of *Mannheimia haemolytica* ghosts as delivery system for DNA vaccines. In vitro studies showed that bacterial ghosts loaded with a plasmid carrying the green fluorescent protein-encoding gene (pEGFP-N1) are efficiently taken up by APC, thereby leading to high transfection rates (52–60%). Vaccination studies demonstrated that ghost-mediated delivery by intradermal or i.m. route of a eukaryotic expression plasmid containing the gene coding for β-galactosidase under the control of the CMV immediate early gene promoter (pCMVβ) stimulates more efficient Ag-specific humoral and cellular ($CD4^+$ and $CD8^+$) immune responses than naked DNA in BALB/c mice. The use of ghosts also allows modulating the major Th response from a mixed Th1/Th2 to a more dominant Th2 pattern. Intravenous immunization with dendritic cells loaded ex vivo with pCMVβ-containing ghosts also resulted in the elicitation of β-galactosidase-specific responses. This suggests that dendritic cells play an important role in the stimulation of immune responses when bacterial ghosts are used as a DNA delivery system. Bacterial ghosts not only target the DNA vaccine construct to APC, but also provide a strong danger signal, acting as natural adjuvants, thereby promoting efficient maturation and activation of dendritic cells. Thus, bacterial ghosts constitute a promising technology platform for the development of more efficient DNA vaccines. *The Journal of Immunology*, 2004, 172: 6858–6865.

Nucleic acid vaccination has emerged as a powerful technology, which can be applied for the development of either prophylactic or therapeutic vaccines (1). The genes encoding the vaccine Ags are cloned into a eukaryotic expression plasmid, which is generally administered by i.m. injection or via biolistic skin bombardment with a gene gun. Then the biosynthetic machinery of the vaccinee's cell is responsible for the in vivo expression of the corresponding gene. The presence of immunostimulatory motifs in the DNA further contributes to the elicitation of an immune response. However, routine implementation of this approach in humans still does not seem to be feasible. This is mainly due to poor immunogenicity and the requirement of extremely high plasmid dosages. The low efficiency of traditional naked DNA vaccination can be due, at least in part, to the fact that APC are not specifically targeted and the encoded Ag is not delivered in the context of an adequate danger signal.

Bacterial ghosts are a novel nonliving vaccination technology platform, which is based on the conditional expression of the lethal lysis gene *E* from bacteriophage PhiX174 in Gram negatives (2–6). This leads to the formation of a transmembrane tunnel through the bacterial cellular envelope (Fig. 1*D*) (2). Due to the high internal osmotic pressure, the cytoplasm content is expelled through the tunnel (see Fig. 1*D*), resulting in an empty bacterial cell envelope (7). Bacterial ghosts retain all morphological, structural, and antigenic features of the cell wall and can be used as vaccine candidate per se. Alternatively, they can be exploited as a delivery system for proteins, which are either expressed and anchored to the envelopes before lysis or subsequently loaded (8). Bacterial ghosts can target APC and microvascular endothelial cells (9–11). The envelope components might provide a danger signal through the activation of pattern recognition receptors, thereby acting as natural adjuvants (12). However, the endotoxic effects of free LPS are not observed, because the LPS is associated to the ghost envelopes (13).

In this study, we evaluated the capacity of *Mannheimia haemolytica* ghosts as delivery system for DNA vaccines. In vitro studies demonstrate, for the first time, that ghosts are efficiently taken up by APC, thereby leading to high transfection efficiencies. Ghost-mediated DNA delivery resulted in the elicitation of more efficient immune responses than using naked DNA, allowing also modulation of the obtained immune response from a mixed Th1/Th2 to a more dominant Th2 response pattern. Intravenous immunization with dendritic cells (DC)[4] loaded ex vivo with plasmid-containing ghosts also resulted in the elicitation of specific humoral and cellular immune responses. Further in vitro studies demonstrated that bacterial ghosts promote efficient maturation and activation of DC. Thus, bacterial ghosts act as natural adjuvants, constituting a promising technology for the development of DNA vaccines.

Materials and Methods

Production and loading of M. haemolytica *ghosts*

Bacterial ghosts were produced by controlled expression of the lysis protein E in the *M. haemolytica* strain A23 (14–16). In brief, electrocompetent

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[4] Abbreviations used in this paper: DC, dendritic cells; EGFP/cgb, enhanced green fluorescent protein; i.d., intradermal

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Bacterial Ghosts as Novel Efficient Targeting Vehicles for DNA Delivery to the Human Monocyte-Derived Dendritic Cells

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Summary: Recombinant bacterial ghosts loaded with plasmids were tested as an antigen delivery system and as a potential mediator of maturation for human monocyte-derived dendritic cells (DCs). Bacterial ghosts are cell envelopes derived from Gram-negative bacteria; the intracellular content is released by the controlled expression of plasmid-encoded lysis gene E of PhiX174. All the cell surface structures of the native bacteria, including the outer membrane proteins, adhesins, LPS, lipid A, and peptidoglycans, are preserved. Co-incubation of immature DCs with ghosts resulted in decreased expression of CD1a, CD80, and CD83 molecules, while addition of maturation mix (TNF-α, IL-1β, IL-6, and PGE_2) to the cultures enhanced expression of these molecules. No marked changes were observed in the expression of the CD1e, CD40, and CD86 surface molecules. The exposure of DCs to ghosts in combination with maturation mix resulted in a nonsignificant increase in their ability to activate T cells. DCs co-incubated with bacterial ghosts carrying plasmids encoding GFP in combination with maturation mix exhibited high expression levels of GFP (up to 85%). These results indicate that in addition to their well-established use as vaccines, bacterial ghosts can also be used as carriers of nucleic acid-encoded antigens.

Key Words: dendritic cells, bacterial ghost, DNA carrier, gene expression

(*J Immunother* 2005;28:136–143)

Dendritic cells (DCs) are the most potent professional antigen-presenting cells (APC) as well as potent initiators and modulators of T-cell response in vivo, including sensitization of MHC-restricted T cells, development of T cell-dependent antibody production, and induction of immunologic tolerance.[1,2] DCs in the periphery capture and process antigens, express lymphocyte co-stimulatory molecules, migrate to the lymphoid organs, and secrete cytokines to initiate immune responses.[3] During their life cycle they change their phenotype from an immature state to mature cells. After the uptake and the processing of antigen (Ag), DCs become functionally mature and acquire the capacity to traffic to the T-cell areas of the secondary lymphoid organs, where they activate Ag-specific naive T cells.[4] Mature DCs (mDCs), representing less than 1% of the mononuclear cell population, are a relevant source of cytokines affecting the development of T-cell response.[5,6] Immature DCs (iDCs), prepared in vitro by cultivation of peripheral blood monocytes in the presence of GM-CSF and IL-4 or IL-13,[6–11] can be further differentiated in vitro into mature-stage DCs by the addition of cytokines such as TNF-α, IL-1β, IL-6, PGE_2, or other agents.[12–14]

DCs represent a potential tool for immunotherapy because of their high ability to present specific antigens to naive T cells and induce immune response.[15] Gene therapy, including genetically modified DCs, is a promising technology in the development of potentially effective vaccines. The successful delivery of antigen or DNA to the target cells (eg, DCs) requires adequate antigen formulation and a suitable delivery system. DNA vaccines consist of bacterial plasmids that code for specific antigens under the control of strong eukaryotic promoters. The expression of a delivered gene should induce strong immune responses or change the behavior of targeted cells. Several delivery systems, including viral vectors with high transfection efficiencies or "safer" nonviral systems such as attenuated bacteria, polycation/DNA complexes, nucleoporation, but with reduced transfection efficiencies, have been described.[20–26] The use of attenuated viral or bacterial systems still poses safety concerns due to their inherent tendency to revert to pathogenicity. The novel recombinant bacterial ghost system represents a new platform in vaccine development. These cell envelopes of Gram-negative bacteria are produced by the controlled expression of lysis gene E, which causes cytoplasmic material to be expelled through the lysis tunnel.[27] Bacterial ghosts retain all morphologic, structural, and antigenic features of the native cell wall and are alternative candidates to live or attenuated bacteria in vaccine development. The inherent adjuvant properties of bacterial ghosts can be attributed to the presence of immune stimulating compounds, such as LPS, peptidoglycans, and Lipid A, on the bacterial surface, and they share the properties of adjuvants because of the possibility of loading the bacterial space with antigens, plasmids, or chemical agents.[28–30]

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ARTICLE

DNA-Loaded Bacterial Ghosts Efficiently Mediate Reporter Gene Transfer and Expression in Macrophages

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There is a demand for efficient and safe DNA delivery vehicles mediating gene transfer and expression. We present bacterial ghosts as a novel platform technology for DNA delivery and targeting of macrophages. Bacterial ghosts are cell envelopes of gram-negative bacteria that are devoid of the cytoplasmic content. *Escherichia coli* ghosts were loaded with plasmid DNA and linear double-stranded DNA. Confocal laser scanning microscopy and flow cytometry confirmed that the DNA localized to the inner lumen of bacterial ghosts and was not associated with the outer surface of the bacteria. Up to ~6000 plasmids could be loaded per single ghost and the amount of loaded DNA correlated with the DNA concentration used for loading. *E. coli* ghosts loaded with plasmids encoding the enhanced green fluorescent protein (EGFP) targeted efficiently murine macrophages (RAW264.7) and mediated effective gene transfer. The EGFP was expressed by more than 60% of the macrophages as measured by flow cytometry detecting the green fluorescence and immunocytochemical staining with antibodies specific for EGFP.

Key Words: bacterial ghosts, DNA delivery vehicle, RAW264.7, macrophages, EGFP expression, transfection

INTRODUCTION

DNA as a therapeutic drug for gene therapy and vaccination is one of the most striking innovations in medical and veterinary sciences. However, the pharmaceutical application of DNA vector systems requires the transfer and the enhanced expression of the DNA-encoded protein, e.g., antigen [1,2]. Currently, viral and nonviral delivery systems are used [3]. Due to the ease of production, the lower toxicity, and the higher biological safety profile, nonviral delivery systems are likely to be favored in DNA vaccination and gene therapy [3,4]. These include polyplexes, lipoplexes, or lipopolyplexes; microparticles; cochleates; and attenuated live bacteria [4,5].

Here, we describe and characterize a novel nonviral DNA delivery and targeting vehicle, bacterial ghosts. Bacterial ghosts are nondenatured gram-negative bacterial cell envelopes devoid of cytoplasmic content and produced by the controlled expression of the plasmid-encoded φX174 gene *E* [6]. Through the created transmembrane tunnel structure bacterial ghosts can be filled with proteins, drugs, DNA, and other water-soluble substances [7,8]. Bacterial ghosts efficiently target antigen-presenting cells [8–10] and other eukaryotic cells [11]. They are, therefore, potential carrier and targeting vehicles for DNA and drugs [12,13]. As a nonviral and nonliving delivery system with the capacity to be loaded with DNA, they are a safe alternative to liposomal and other particulate carrier systems.

Macrophages are involved not only in the resolution of injuries and tissue remodeling [14] but also in the progression and onset of various diseases, including the growth and spread of, for example, malignant tumors, HIV infection, and inflammation in rheumatoid arthritic joints [5]. Macrophages have been proposed as cellular delivery vehicles for adoptive immunotherapy, as they localize to sites of inflammation and tumors, adhere to the endothelium, and transmigrate to the focus of injury [14]. Potential applications of macrophage transfection include gene-dependent enzyme

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Exhibit F